Ciglitazone reverses cAMP-induced post-insulin receptor resistance in rat adipocytes in vitro

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Ciglitazone (cig), a thiazolidine-dione, lowers glucose and insulin levels in animal models of diabetes type II but not in controls. Since catecholamines given to rat adipocytes in vitro induce insulin resistance similar to that seen in type II diabetes in vivo, we measured the effect of cig on mono-A14-[^{125}I]insulin binding and 3-O-methyl-D-glucose transport (GT) in isolated rat adipocytes treated with isoprenaline (iso, 10 μM). Cig ($\leqslant$ 5 μM) reversed (ED_{50} 10 nM) the inhibitory effect of iso on insulin stimulation of GT. It had no effect on either basal or insulin stimulated GT. Furthermore, cig did not influence insulin binding either in the presence or absence of iso, which indicates that cig acts only on a post-insulin receptor level. Cig also reversed the inhibiton of GT by both forskolin, a cyclase activator and RO20-1724, an imidazolidine phosphodiesterase inhibitor but not that of db-cAMP. It thus seems that cig does not act within the cAMP system but only neutralizes its inhibitory effect on the insulin stimulation of GT.

Insulin receptor *Diabetes* *Insulin resistance* *Ciglitazone* *Glucose transport* *cAMP*

1. INTRODUCTION

Ciglitazone has been reported to be beneficial in animal models of type II diabetes, which are characterized by resistance to insulin therapy, by decreasing hyperglycemia, hypertriglyceridemia and hyperinsulinemia and improving the insulin tolerance test [1,2]. It had no effect in animal models of insulinopenic diabetes or normal animals [1,2] or on the β-cells of the pancreas [1]. The improvement of the diabetic symptoms in type II diabetes seems to be mainly due to an effect on carbohydrate metabolism. Thus, in a study of glucose disposal in ob/ob mice, glucose turnover was doubled, gluconeogenesis decreased and glucose incorporation in hepatic lipids, glycogen and protein was increased in the ciglitazone-treated animals [3]. Glucose oxidation and incorporation in lipids were also increased and potentiated by insulin in adipocytes from ciglitazone-treated ob/ob mice [4]. Insulin sensitivity and responsiveness were improved, so that in addition to the observed increase in insulin binding an improvement of a post-insulin receptor resistance in the diabetic mice was postulated [4]. In ciglitazone-treated obese Sprague Dawley rats, however, glucose oxidation was increased in fat pads but no effect on insulin binding was observed. This confirmed the effect on glucose metabolism and indicated a direct ciglitazone effect on a post-insulin receptor resistance independent from insulin binding [5].

We showed recently that isoprenaline inhibits insulin binding and the insulin stimulation of D-glucose transport in isolated rat adipocytes [6] via a cAMP-dependent mechanism which is probably a post-insulin receptor mechanism since it was mainly the insulin responsiveness of D-glucose transport which was lowered [7]. These isoprenaline effects have been reported by other

Abbreviations: ciglitazone, 5-[4-(1-methylcyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione; cAMP, adenosine cyclic 3′,5′-monophosphate; db-cAMP, N^6,O^2-dibutyryl-cAMP; RO20-1724, d-1,4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone

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groups too [8,9] and offered a model for the study of the post-insulin receptor resistance seen in type II diabetes. Here we investigated in vitro the effect of ciglitazone on cAMP-induced insulin resistance in isolated rat adipocytes.

Our results indicate that ciglitazone acts mainly by reversing the inhibitory effect of isoprenaline on insulin stimulation of the translocation of the glucose transporters into the cell membrane.

2. MATERIALS AND METHODS

Fat cells were prepared from male Sprague-Dawley rats fed ad libitum (180–220 g body weight). Krebs–Ringer–Hepes buffer (pH 7.4, 37°C) containing 2.5 g/dl crystalline bovine serum albumin was used for all incubations. High cAMP levels were achieved as described in [6,7] by a 30-min preincubation with isoprenaline which was carried out in the presence of 16 mM D-glucose to stabilize intracellular levels of ATP [6]. Ciglitazone was prepared according to [10]. In the experiments in which the ciglitazone effects after insulin stimulation were studied, isoprenaline (10 μM, Serva, Heidelberg) and adenosine deaminase (10 U/ml, Sigma, Munich) were added 20 min after insulin (0.67 nM) as described in [11]. Forskolin was obtained from Calbiochem, Frankfurt, RO20-1724 from Hoffmann-LaRoche, Grenzach, metformin from Chemiewerk Homburg, Frankfurt, and gliquidone from Dr K. Thomae GmbH, Biberach.

2.1. *3-O-Methylglucose transport*

3-*O*-Methylglucose transport was measured as described in [14]. Aliquots (100 μl) of the concentrated cell suspension (5×10^6 cells/ml) were drawn together with 200 μl 3-*O*-methyglucose (final concentration 0.5 mM) and a tracer of 0.1 μCi 3-*O*-methyl-D-[^{14}C]glucose (Radiochemical Centre, Amersham) into a mixing pipette (Gilson Medical Electronics, France). After 4 s, the uptake was stopped by diluting the cells in 5 ml NaCl (0.9 g/dl) which contained phloretin (1 mM). Cells and medium were separated by centrifugation through silicone oil ($1000 \times g$ for 60 s). The cell layer was removed by a pipette, added to scintillation fluid, and the radioactivity measured. The amount of 3-*O*-methylglucose in extracellular fluid or taken up by diffusion was determined in samples which contained 1 mM phloretin. All other uptake data were corrected by this value. 3-*O*-Methylglucose accumulation measured during 4 s is expressed as a percentage of the 3-*O*-methylglucose accumulated at equilibrium in fat cells after 30 min incubation at saturating insulin concentrations (6.7 nM).

2.2. *Binding studies*

After preincubation of fat cells ($4.5–5.5 \times 10^5$ cells/ml) 33 or 67 pM mono-A14-[^{125}I]insulin (specific activity 250 μCi/μg, Novo Industrie, Bagsvaerd, Denmark) were added alone or together with unlabelled insulin in concentrations between 0.1 and 6.7 nM. After 20 min, 400-μl aliquots were transferred into polyethylene centrifuge tubes and the cells were separated from the medium by centrifugation through dinonylphthalate [14]. The tubes were cut at the oil layer and radioactivity of the cell layer was measured. Non-specific binding was assessed by addition of unlabelled insulin (6.7 μM) to the tracer and the value was substracted.

For statistical evaluations the two-tailed Student's *t*-test for paired observations was used.

3. RESULTS AND DISCUSSION

3.1. *Ciglitazone effects on isoprenaline-induced insulin resistance*

As described earlier [6], a 30-min preincubation with isoprenaline (10 μM) inhibits the insulin stimulation of D-glucose transport by 45.6 ± 5.1% in isolated rat adipocytes at insulin concentrations exceeding 0.17 nM (fig.1). The ED_{50} of insulin (0.61 nM) was not changed. In cells treated for 30 min with isoprenaline as above and ciglitazone (5 μM) the glucose transport values were almost equal to control values at the same insulin concentrations (fig.1). Sensitivity of glucose transport to insulin remained unchanged, ciglitazone only reversing the decrease in insulin responsiveness caused by isoprenaline. Ciglitazone did not alter the basic D-glucose transport (fig.1), suggesting that its effect is insulin dependent. The reversal of the isoprenaline inhibition of insulin stimulation of D-glucose transport by ciglitazone was concentration dependent (ED_{50} 10 nM) and maximal at about 1 μM (fig.2). When given with insulin alone ciglitazone had no effect (fig.2). These results sug-

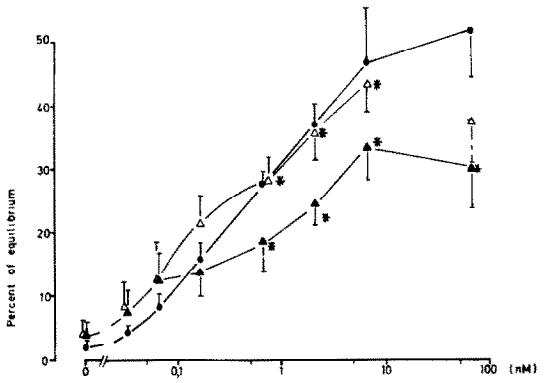

Fig.1. Effect of ciglitazone on the isoprenaline-induced inhibition of insulin stimulation of D-glucose transport in isolated rat adipocytes. Epididymal rat adipocytes were isolated with the collagenase method and incubated in Krebs–Ringer–Hepes buffer (37°C, 16 mM glucose, pH 7.4) in the absence (●) or presence of isoprenaline (10 μM, (▲, △) and ciglitazone (cig, 5 μM, △) for 30 min. Then insulin was added in concentrations between 30 pM to 67 nM to aliquots, and D-glucose transport was measured after 20 min with 3-*O*-methylglucose in 4 s as described in section 2 and expressed as a percentage of the equilibrium space filled in 30 min. The data represent the mean of 5 independent preparations measured in duplicate. D-Glucose transport after preincubation with isoprenaline was significantly lower (* $P < 0.04$) than control at insulin concentrations exceeding 0.17 nM. The glucose transport after preincubation with isoprenaline and ciglitazone was significantly higher (* $P < 0.05$) than with isoprenaline alone at insulin concentrations between 0.17 and 67 nM. Statistical significance was calculated using the two-tailed Student's *t*-test for paired observations.

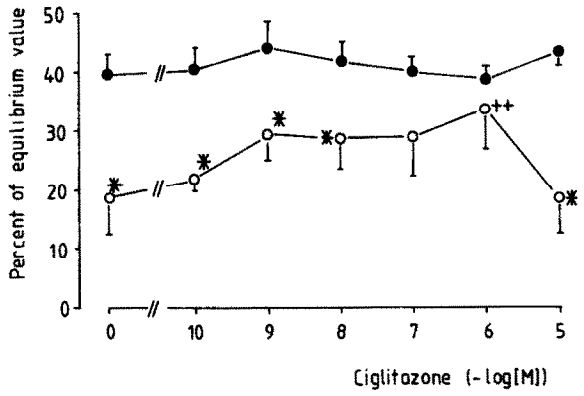

Fig.2. Concentration dependence of the ciglitazone effect on the isoprenaline-induced inhibition of insulin stimulation of D-glucose transport in isolated rat adipocytes. Epididymal rat adipocytes were isolated with the collagenase method and preincubated in Krebs–Ringer–Hepes buffer (37°C, 16 mM glucose, pH 7.4) in the presence (○—○) or absence (●—●) of isoprenaline (10 μM). The aliquots contained ciglitazone in concentrations between 0 and 1 μM as indicated on the abscissa. After 30 min, insulin (2 nM) was added and D-glucose transport measured after 20 min as described in section 2. The data represent the mean ± SE of 7 independent preparations, each measured in duplicate. The D-glucose transport in isoprenaline-treated cells was smaller than in controls until a ciglitazone concentration of 10 nM (* $P < 0.05$) was reached. At 1 μM ciglitazone, the D-glucose transport was higher than the value obtained with isoprenaline alone ($^{++}P < 0.01$). Statistical significance was calculated using Student's two-tailed *t*-test for paired observations.

gest that even though its action is insulin dependent, ciglitazone is not a general potentiator of insulin action [5].

Moreover, this effect seemed to be specific for ciglitazone, as other oral anti-diabetic agents like metformin (4 μg/ml) or gliquidone (10 μg/ml) did not remove the inhibition of insulin stimulation of glucose transport caused by preincubation with isoprenaline (10 μM) for 30 min ($n = 6$, not shown).

As isoprenaline decreased insulin binding as well [6,8], we investigated whether ciglitazone also reversed this effect. As shown in fig.3, after 30 min preincubation isoprenaline (10 μM) lowered insulin binding by 33 ± 8% at insulin concentrations below 2 nM [6]. This decrease of high affinity binding [6] was not reversed by ciglitazone (5 μM, fig.3). It seems therefore that ciglitazone only counteracts the insulin resistance at post receptor level.

Our results thus agree fully with the ones obtained with ciglitazone-treated obese Sprague-Dawley rats [5], where an enhancement of insulin induced glucose oxidation but no effect on insulin receptors were found. They agree only partly with the ones obtained with adipocytes of treated ob/ob mice [4], where increased insulin-induced glucose oxidation and incorporation in lipids were reported together with an increase in insulin binding. The increase in insulin binding observed in these

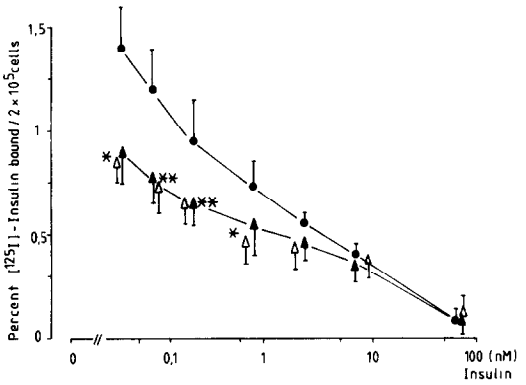

Fig.3. Effect of ciglitazone on the isoprenaline-induced inhibition of insulin binding in isolated rat adipocytes. Cells were treated as in fig.1 and binding of mono-A14-[^{125}I]insulin was determined as described in section 2. On the ordinate, the fraction of [^{125}I]insulin (%) which bound on addition of unlabelled insulin as indicated on the abscissa is given. The data represent the mean ± SE of 4 independently performed experiments measured in duplicate. Ciglitazone had no effect on the isoprenaline-induced inhibition of binding between 0.03 and 0.67 nM insulin. Significance was calculated using the two-tailed Student's *t*-test for paired observations (**$P < 0.01$).

experiments might very well have been due to the decrease of hyperinsulinemia which followed the improvement of the post-insulin receptor resistance brought about by ciglitazone and could also account for the increase of insulin sensitivity which was observed in these experiments but not in ours. On a more general scale, glucose transport is thought to be rate-limiting for glucose metabolism in adipocytes. Our finding that ciglitazone reverses post-insulin receptor resistance by counteracting the inhibitory effect of catecholamines on insulin stimulation of glucose transport could therefore explain the improvement in glucose metabolism caused by ciglitazone in vivo [1–5].

3.2. *Possible site of interaction of ciglitazone with the glucose transport system*

Having found that ciglitazone removed the inhibition of glucose transport by isoprenaline, we went on to investigate how it interacts with the glucose transport system. Isoprenaline inhibits insulin stimulation of D-glucose transport by two main mechanisms [6,9]: by inhibiting the translocation of glucose transporters from an inner compartment to the cell membrane and by changing the affinity of the glucose transporters in the cell membrane. When given before insulin, isoprenaline lowers the V_{max} of glucose transport, which means that a smaller number of glucose transporters are present in the cell membrane [6,9]. When given on the insulin stimulated glucose transport, isoprenaline should, according to [9], have no effect on translocation, as more than 50% of the intracellular glucose transporters are then already translocated to the cell membrane causing an 8-fold increase of glucose transport [12,13].

In our experiments, we added isoprenaline (10 μM) and adenosine-deaminase (10 U/ml) 20 min after insulin (0.67 nM) for 30 min according to [11]. Glucose transport was then measured and a significant decrease of glucose transport by 54.3 ± 19.5% was observed ($n = 4$, $P < 0.01$). When ciglitazone (1 μM) was added together with isoprenaline and adenosine deaminase, the glucose transport value improved by 10% which was not significant ($P < 0.02$). This glucose transport value was still significantly lower than the control value ($P < 0.01$, $n = 4$). Since ciglitazone did not reverse the isoprenaline effect on insulin stimulated glucose transport, one could conclude that it does not influence the isoprenaline effect on the affinity of the transporters in the cell membrane but probably acts by reversing its inhibitory effect on translocation of glucose transporters.

3.3. *Possible site of ciglitazone interaction with the cAMP system*

Since the isoprenaline-induced resistance of glucose transport has been shown to be mediated by cAMP [7], we investigated the ciglitazone effect on the cAMP system, even though it is not known whether cAMP has a role in the insulin resistance of type II diabetes.

Ciglitazone (1 μM) was given to isolated rat adipocytes together with various cAMP modulating substances which act at specific points of the cAMP system. We reasoned that if ciglitazone interferes with the catecholamine receptor or the regulatory subunits of the adenylate cyclase then it should have no effect in the presence of forskolin, an agent which directly stimulates the catalytical subunit of the adenylate cyclase. If ciglitazone acts by stimulating the phosphodiesterase and thereby lowering cAMP, then a phospho-

diesterase inhibitor like RO20 should prevent the ciglitazone effect. If, however, the ciglitazone action should take place at or after the cAMP-stimulated protein kinase, then even the effect of cAMP analogues like db-cAMP should be antagonized. As shown in table 1, all the above-mentioned substances inhibited the insulin stimulation of glucose transport to a significant degree. Ciglitazone antagonized all of them except db-cAMP, which means that it could overcome an increase in endogenous cAMP but was not effective against the exogenously added db-cAMP. This could indicate that ciglitazone is a direct competitor of cAMP on the cAMP-dependent protein kinase. This is however unlikely as ciglitazone should then antagonize all cAMP effects and also reverse the cAMP-induced inhibition of insulin binding and of glucose transport after insulin stimulation. The interaction with the cAMP system thus seems to be limited to the reversal of cAMP-mediated inhibition of the translocation of glucose carriers to the cell membrane.

Evidence for an interaction of ciglitazone with cAMP in glucose metabolism has also been obtained in vivo. Thus diabetic ob/ob mice that lacked the 20–25% inhibition of glucose 1-^{14}C oxidation after epinephrine which was observed in control groups reacted to epinephrine like the controls when they were given ciglitazone [4]. Ciglitazone, however, had no effect on epinephrine responsive metabolic processes like lipolysis in isolated fat pads of either lean or obese mice [4] in vitro, or on the epinephrine-induced hyperglycemia and lipolysis in ob/ob mice and controls in vivo [2].

In conclusion, we showed that ciglitazone reversed the isoprenaline-induced insulin resistance in isolated rat adipocytes as it improved glucose metabolism in the animal models of type II diabetes in vivo [1–5]. It seems to interfere with the cAMP-mediated inhibition of the translocation of glucose carriers to the cell membrane, thus allowing insulin stimulation of glucose transport almost as in the absence of cAMP inhibition.

Table 1

Effects of ciglitazone on the cAMP-induced inhibition of insulin stimulation of D-glucose transport

Insulin (2 nM) + addition	D-Glucose transport (%)		Δ^{1a}	P
	Without	With ciglitazone		
–	43.5 ± 6.9	45.8 ± 7.6	2.3 ± 2.8	n.s.
Isoprenaline	20.1 ± 2.3	32.1 ± 2.8	12.0 ± 3.6	≅0.01
Forskolin	18.5 ± 5.0	24.7 ± 4.6	6.1 ± 0.6	≅0.01
RO20	30.6 ± 4.0	38.5 ± 3.5	7.9 ± 2.2	≅0.01
db-cAMP	21.9 ± 0.7	22.2 ± 2.4	0.3 ± 0.1	n.s.

[a] Δ^1, Difference between absence and presence of ciglitazone

Epididymal rat adipocytes were isolated with the collagenase method and incubated in Krebs–Ringer–Hepes buffer (37°C, 16 mM glucose, pH 7.4) in presence or absence of ciglitazone (1 μM) with isoprenaline (10 μM), forskolin (0.1 mM), RO20 (RO20-1724, 1 mM) and db-cAMP (1 mM) for 30 min and then insulin (2 nM) was added. After 20 min, D-glucose transport was measured in 4 s and expressed as percent of equilibrium uptake of 3-*O*-methylglucose. The data represent the mean ± SE of 5 independent preparations each measured in duplicate. The aliquots which contained cAMP-modulating substances and no ciglitazone all had lower glucose transport values ($P < 0.01$; RO20: $P < 0.05$) than the aliquots containing only insulin. When compared to the ones also containing ciglitazone, the latter were significantly ($P \cong 0.01$) higher, except for the aliquot containing db-cAMP or insulin alone. Statistical significance was evaluated using the two-tailed Student's t-test for paired observations

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